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A Study to Sustain the Hypothesis of the Multiple Genesis of Oligoasthenoteratospermia in Human Idiopathic Infertile Males

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ABSTRACT

Interdependence between sperm concentration, motility, morphology, and the percentage of aneuploid sperm was explored to test whether oligoasthenoteratospermia (OAT) may have a multiple origin in idiopathic infertile males. A total of 174 men (age, 35.8 ± 4.3 yr) with idiopathic infertility were studied. Seven patients had nonobstructive azoospermia, 55 had severe OAT, 30 had OAT, 27 had isolated alterations of motility, 45 had alterations of morphology and of motility, and 10 had isolated alterations of morphology. The sperm morphology was assessed with strict criteria. The percentage of aneuploid sperm was assessed with fluorescent in situ hybridization for chromosomes X, Y, 13, 15, 16, 17, 18, 21, and 22. Relationships between sperm features, and the relationship between sperm features and aneuploidies were analyzed with multivariate regression analysis. Statistical analysis did not find any significant relationship between the percentage of typical forms and sperm concentration or between morphology and motility. On the other hand, a positive and significant relationship was found between sperm concentration and motility. The percentage of aneuploid sperm was inversely and significantly related to the percentage of typical forms but not to motility and concentration. Sperm morphology is an independent characteristic with respect to concentration and motility, whereas it showed a significant inverse relationship with respect to the percentage of aneuploid sperm. This means that idiopathic OAT may occur by means of at least two independent pathways, the first affecting concentration and/or motility and the second affecting morphology.

gamete biology, gametogenesis, idiopathic oligoasthenoteratospermia, male infertility, sperm

INTRODUCTION

Idiopathic (oligo-)asthenoteratozoospermia (iOAT) is defined as defective spermatogenesis in which conventional sperm analysis is altered, but the common clinic and laboratory parameters are within the normal range; iOAT affects approximately 30% of OAT infertile men [1]. Three mechanisms have been quoted to explain iOAT pathogenesis: 1) increased reactive oxygen species produced by immature gametes in the tubula and in seminal plasma [2], 2) spontaneous modified expression of genes regulating programmed cell death (PCD) [3, 4], and 3) modifications of mitochondrial DNA [5, 6]. Therefore, it has been postulated that iOAT may result from infertilities of different origins [7]; however, there is no direct proof that the cohort of iOAT infertile patients is not homogeneous in terms of iOAT genesis. This paper is aimed at ascertaining whether a multiple origin of iOAT can be demonstrated in a cohort of iOAT infertile males.

Sperm morphology, density, and motility should be considered as the final product of spermatogenesis [8]; we therefore hypothesized that the multiple genesis of iOAT might be looked for in conventional seminal analyses with normal physical semen characteristics (i.e., appearance, consistency, liquefaction, volume, and pH [9]). Using a prospective correlational design, we investigated whether relationships between sperm morphology, concentration, and/or motility exist in iOAT males, assuming that any lack of relationship among sperm features might be regarded as proof that more than one mechanism can lead to iOAT. Research such as this could be carried out through mere statistical evaluation, but it would entail the risk of assessing mathematical relationships rather than the real biological ones. Biological relationships must be detected in order to validate our hypothesis; therefore, relationships among sperm features and other sperm tests were examined here. Sperm function tests are difficult to interpret, and it is still a struggle to clarify the complex nature of the molecular interactions between sperm and eggs, and the processes of capacitation and acrosome reaction [10]. On the other hand, an attempt to link sperm morphology, motility, and concentration to the percentage of aneuploid sperm assessed with fluorescent in situ hybridization (FISH) seems to be relevant for a number of reasons: first, the percentage of aneuploid sperm is inversely related to the quality of spermatogenesis [11–13], and second, the etiology of aneuploidy (i.e., meiotic spindle alterations) can be identified in the FISH sample [14].

MATERIALS AND METHODS

Patients

The study was authorized by the Società Italiana di Studi di Medicina della Riproduzione (SISMER) Institutional Review Board, and each couple signed an informed consent form. In the present study, we prospectively analyzed the sperm parameters, including the aneuploidies of patients attending a private infertility clinic (SISMER) from 1 April 2004 to 30 July 2005. Each man contributed two sperm specimens, one of which was used to test the aneuploidies.

All patients whose chief complaint was primary couple infertility associated with oligospermia and/or asthenospermia and/or teratospermia [15] were considered for inclusion in the study. A total of 443 patients were visited (age, 38.2 ± 5.9 yr); of these, only patients affected by iOAT were studied. This

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term, *iOAT*, indicates male factor infertility characterized by an oligospermia and/or asthenospermia and/or teratospermia and no alteration of the common clinical, instrumental, and laboratory parameters. The following assessments were carried out for iOAT diagnosis. All patients underwent anamnesis, sexological counselling session(s), physical examination (i.e., general inspection of the body, evaluation of sexual characteristics, muscular mass, fat and hair distribution, and evaluation of pubic hair according to Tanner stages, inspection and palpation of the genitalia, and prostate rectal palpation [16]). All patients had their serum levels of total prostatic serum antigen, FSH, LH, prolactin, and total and free testosterone assessed [15].

Patients with a sperm concentration of less than 5×10^6 cells/ml had their chromosomes analyzed and Y microdeletion assessed. Potential cystic fibrosis was detected in patients with nonobstructive azoospermia, and those men were examined for any prostatic median cysts (using transrectal ultrasound); the nonobstructive nature of the azoospermia was confirmed on surgical specimens collected during the course of testicular sperm extraction (TESE) [17].

If one or more of the following criteria were present, the patients were not considered iOAT and were eliminated from the present study: abnormal physical semen characteristics (38 cases); chromosomal alterations (six cases); seminal white blood cell concentration >1 000 000/ml and/or positive seminal cultural analysis and/or positive urethral swab chlamydia test (37 patients); hormonal alterations (39 patients with elevated FSH [>10 IU/l]); age older than 40 yr (20 patients); the presence of anti-sperm antibodies (tested both in sera and bound to the sperm surface; 18 patients); drug, tobacco, or alcohol abuse (28 patients); ongoing medical treatment (gonadotropins, anabolic steroids, cancer chemotherapy, nonsteroidal anti-inflammatory drugs; six patients); the presence of a hydrocele (nine patients); diabetes (six patients); hypertension (six patients); x-ray exposure in the previous 8 mo (three patients); varicocele (59 patients); and prostate abnormalities found with digital rectal exploration or suprapubic echography (29 cases). Prostate abnormalities are intended as: volume >20 cm^3 and/or tenderness and/or modifications of consistency found with digital rectal exploration and modifications of echogenicity found with a suprapupic echoscan and/or total prostatic specific antigen higher than 4 μg/ml. Other exclusion criteria used were previous or concurrent testicular pathology (torsion, undescended testicle, orchiepididymitis, surgery, trauma, or neoplasm) and/or testicle echographic abnormalities (intended as focal or diffuse change of testicle structure and/or volume; three cases) [16]. In total, 209 patients were invited to participate in the study; 19 refused and 190 accepted.

Semen Analysis

Each patient delivered two semen samples by masturbation [9]. Each was assessed according to World Health Organization guidelines [9], and the mean value of the two was used for calculations [8]. Written informed consent was obtained from all participants after recruitment. Semen samples were collected by masturbation at the laboratory after a 72-h abstinence from ejaculation. The two sperm samples were collected at a distance of 7–30 days from one another [9]. All semen analyses were carried out manually within 1 h after the semen was collected in the laboratory. The semen samples were left to liquefy at 37°C for 20 min. Immediately after liquefaction, a drop of the well-mixed specimen was placed on a clean glass slide, covered with a coverslip, and left for a few minutes. The preparation was examined under a magnification of both 10× and 40× objectives.

Motility assessment was done on two samples from the same ejaculate observing at least 200 spermatozoa in each, using at least 10 randomly selected, separated high-power fields. The motility of each sperm was graded A, B, C, or D according to whether it showed (A) rapid progressive motility, (B) slow or sluggish progressive motility, (C) nonprogressive motility, or (D) immotility [18]. Each ejaculate was evaluated for sample variability with Pearson coefficients. Since values >0.90 and <0.93 were obtained, the differences between the two samples did not need any further analyses [19].

Depending on the estimated sperm concentration, a 1:10, 1:20, or 1:100 dilution of the semen sample was made with the use of a glass tuberculin syringe using Ham F10 + 10% BSA medium. An improved double-rule Neubauer hemocytometer was used for counting the spermatozoa. Two dilutions were made for every sample. The difference between the two dilutions was not greater than 10% for low concentrations and not greater than 20% for concentrations $>60 \times 10^6$/ml [18].

To assess the morphology, two semen smears from each patient were stained at the laboratory using the Papanicolaou method and assessed for sperm morphology by a single technician. Either 200 or 300 sperm were analyzed per slide. Initially, 200 sperm from each of two different locations on the slide were analyzed. If the difference between the percentages of normal sperm in the two tested areas was five percentage points or less, the mean value was calculated. If the difference was more than five percentage points, an additional 200 sperm were evaluated from a third location on the slide, and the median of all three values was used. The mean value of the two smears was used [20]. In this laboratory, a spermatozoon was considered normal when the head had a smooth, oval configuration and a well-defined acrosome comprising about 40%–70% of the sperm head. There also had to be no neck, midpiece, or tail defect and a cytoplasm droplet of more than one half the size of the sperm head. Borderline forms were considered abnormal [21, 22]. Counted gametes were classified into one of seven groups: normal (head and tail normal), normal head but with another abnormality present, large heads, small heads, tapering heads, duplicated heads, or amorphous heads, all with or without tail, neck, or midpiece defects. Tail, neck, and midpiece defects, loose head, immature gametes, white blood cells, epithelial cells, and unknown cells were recorded separately. Samples from nonobstructive azoospermic patients were considered as having a zero spermatozoa/ml concentration.

FISH Procedure

A minimum of 5000 spermatozoa per hybridization per subject were examined for aneuploidies [13]; 16 of 190 patients could not achieve this concentration, and they were not considered in the present study.

Semen Sample Collection

Fluorescent in situ hybridization analysis was carried out on one of the two semen samples collected from each patient, according to the availability of the technicians specialized in FISH analysis. Fluorescent in situ hybridization analysis of one semen sample was regarded as sufficient because a nonsignificant intraindividual variability was observed in the course of 15 FISH tests (Pearson coefficients: >0.90, <0.93 [19]). Our data have been confirmed by the current literature; therefore, FISH should be regarded as a reproducible test [23, 24]. These data led us to believe that our procedure did not systematically bias our results. Semen samples were fixed immediately after collection. Seven samples from nonobstructive azoospermic patients were collected with TESE; these samples had previously been stored in liquid nitrogen and then thawed and fixed. Spermatozoa retrieved by TESE had been cryopreserved after surgical intervention for subsequent use in assisted reproduction cycles; with the patient's consent, two or three straws were thawed for FISH analysis. Cryopreservation was carried out according to the Martin procedure, which has no effect on sperm FISH [12].

Sperm Nuclei Preparation

The samples were washed three times in PBS (pH 7.2) at $300 \times g$ for 10 min. The pellet was resuspended in 1 ml cold methanol:acetic acid (in proportion 3:1) and stored at −20°C until further processing. The fixed sperm were cytospun on poly-L-Lys-coated slides, washed in 2× saline sodium citrate (SSC) with 0.3 M NaCl, and decondensed in 1 M Tris-HCl (pH 9.5) with 25 mM dithiothreitol for 5 min at room temperature. After rinsing in 2× SSC and PBS, the slides were dehydrated in an ethanol series of increasing concentration.

Cytoplasmic Staining

The sperm morphology data used in the present paper derive from semen analysis; the morphology of the FISH specimens is not presented here. In fact, the procedures for the FISH sample preparations are completely different from the recommended procedures for strict criteria morphology assessment in semen samples. The Papanicolaou method of cytoplasmic staining was used with some modifications, as indicated previously [14].

FISH on Sperm

Multicolor FISH was used in a two-step protocol to diagnose each sperm cell for chromosomes X, Y, 13, 15, 16, 17, 18, 21, and 22, as indicated previously [14]. The probe mixture used in the first round contained the probes specific for chromosomes LS1 13 (RB-1 locus, 13q14 expanding 440 kb), CEP 16 (satellite II D16Z3), CEP 18 (alpha satellite, D18Z1), LSI 21 (region 21q22.13–21q22.2), and LS1 22 (region 22q11.2); the MultiVysion PB Assay produced by Vysis Inc. (Downers Grove, IL) was used. A second round followed, with probes specific for chromosomes X (CEP X alpha satellite, Xp11.1–q11.1), Y (CEP Y alpha satellite, Yp11.1-q11.1), 15 (CEP 15 satellite III, 15q1 1.2), and 17 (CEP 17 alpha satellite, 17p1 1.1–q11.1) obtained from Vysis.

The slides were viewed using an Olympus BX40 fluorescence microscope at 600× magnification (60× immersion lens, 10× ocular) equipped with a Ludl filter wheel equipped with the following filter sets: dual bandpass filters (red/green and aqua/blue; Olympus, Osaka, Japan) and single bandpass filters (red, green, yellow, aqua; Olympus). The probes were labeled as follows:

TABLE 1. Seminal fluid characteristics of the population studied, classified according to WHO in 1999 [9].[a]

Patient	No. of patients	Age	Ejaculated volume	Sperm concentration $\times 10^6$/ml	Percentage of class A motile sperm	Percentage of typical forms	Percentage of aneuploid spermatozoa[b]
NOA[c]	7	34.3 ± 4.0	2.7 ± 1.0	0	0	0	24.1 ± 13.2
sOAT[d]	55	33.9 ± 5.0	2.5 ± 1.6	1.2 ± 1.7	6.3 ± 9.5	3.9 ± 4.5	10.0 ± 14.5
OAT[e]	30	32.7 ± 4.8	2.9 ± 1.5	10.2 ± 3.9	15.6 ± 8.3	10.1 ± 5.1	3.3 ± 4.1
Mt[f]	27	35.0 ± 4.4	2.8 ± 1.4	62.1 ± 35.2	17.8 ± 3.8	16.6 ± 2.5	3.4 ± 6.8
Mf+Mt[g]	45	36.1 ± 3.8	2.7 ± 1.6	53.3 ± 32.4	12.8 ± 6.2	9.6 ± 3.8	3.5 ± 7.2
Mf [h]	10	36.0 ± 3.4	2.9 ± 1.7	54.0 ± 27.4	28.5 ± 3.5	10.7 ± 1.6	2.2 ± 1.6

[a] Values are mean ± SD.
[b] The percentage of aneuploid spermatozoa was calculated by doubling the incidence of disomy.
[c] NOA = nonobstructive azoospermic patients.
[d] sOAT = severe oligoasthenoteratospermic patient (= sperm concentration $<5 \times 10^6$/ml).
[e] OAT = oligoasthenoteratospermic patient (= sperm concentration $>5 \times 10^6$/ml and $<20 \times 10^6$/ml).
[f] Mt = patients with isolated alterations of motility (= sperm concentration $>20 \times 10^6$/ml, WHO class A motile sperm <25%, morphology [strict criteria] >14%).
[g] Mf+Mt = patients with isolated alterations of motility and of morphology (= sperm concentration $>20 \times 10^6$/ml, WHO class A motile sperm <25%, morphology [strict criteria] <14%).
[h] Mf = patients with isolated alterations of morphology (= sperm concentration $>20 \times 10^6$/ml, WHO class A motile sperm >25%, morphology [strict criteria] <14%).

chromosomes X and 16 with Spectrum Aqua (peak of excitation 433 nm, peak of emission 480 nm), 13 with Spectrum Red (peak of excitation 592 nm, peak of emission 612 nm), 15 and 21 with Spectrum Green (peak of excitation 497 nm, peak of emission 524 nm), 18 with Spectrum Blue (peak of excitation 400 nm, peak of emission 450 nm), 17 with Spectrum Orange (peak of excitation 558 nm, peak of emission 734 nm), 22 with Spectrum Gold (peak of excitation 530 nm, peak of emission 555), and Y with Spectrum Aqua and Orange. The X and 16 chromosome signals appear as bright blue, 13 as red, 15 and 21 as green, 18 as blue, 17 as orange, 22 as yellow, and Y as pink. The images were recorded using a Photometric Sensys KAF-1400 CCD camera (Roper Scientific, Tucson, AZ) controlled by a Macintosh (Apple, Cupertino, CA) computer and image analysis software (Vysis Quips, Downers Grove, IL).

Spermatozoa were diagnosed as abnormal if they presented two or more fluorescent signals for the same chromosome whose size and intensity were similar to those detected in normal nuclei. Diploidy was defined as the presence of two signals for each of the chromosomes studied in the presence of the sperm tail, and an oval head shape and sperm were defined as nullisomic when no fluorescent signals appeared.

During microscopic observation, the position of each sperm cell was assessed by the coordinates defined by the graduate scale on the microscope table, allowing us to diagnose each spermatozoon of the nine chromosomes studied by the two rounds of FISH.

Data Management and Statistical Analysis

Evaluation of the incidence of aneuploidy followed a conservative approach by doubling the incidence of disomy [14]. The end points of the current study were: 1) to ascertain whether a significant relationship exists among sperm concentration, motility, and morphology, and 2) to ascertain whether a significant relationship exists between the percentage of aneuploid sperm and each sperm feature (sperm concentration, percentage of class A motile sperm, and percentage of typical forms assessed with strict criteria).

To investigate these end points, multivariate regression analysis was used. Relationships were evaluated for significance using inferential analysis of the regression coefficients of the equations that define the relationships. This research was stopped according to the O'Brian-Fleming stopping boundaries [25] for the following reasons: 1) the result expected was not obtained, 2) nothing was known about the possibility of achieving statistical significance, and 3) this was not a trial but a prospective correlation study [26]. Effectively, the following variables were also examined for regression: number of ejaculated spermatozoa (intended as sperm density × ejaculated volume); number of normal spermatozoa (intended as total ejaculated spermatozoa × percentage of class A motile sperm × percentage of typical forms); and percentage of normal spermatozoa (intended as: [number of normal spermatozoa/number of ejaculated spermatozoa] × 100). Since no significance was found, they were not presented for the sake of brevity.

RESULTS

Table 1 presents the data of the patients studied. Patients were classified by their seminal data according to the World Health Organization nomenclature [9]. There was no meaningful difference in terms of ejaculated volume and age among the classes of patients when examined by variance analysis [25]. A total of 1 012 854 spermatozoa were evaluated for aneuploidies, and the mean per patient was 5821 ± 232 spermatozoa. Each spermatozoon was assessed for all the chromosomes studied (see *Materials and Methods, FISH on Sperm*). Table 2 presents the percentage of aneuplodies of each chromosome studied (in bold) and a comparison with the percentages of aneuploidies of chromosomes which are reported in the literature. Percentage of aneuploid spermatozoa was computed by doubling the incidence of disomy in Tables 1 and 2.

The results of multivariate regression analysis are presented in Table 3. It shows that sperm morphology is not significantly related to concentration and motility and that there is a positive and significant relationship between sperm concentration and sperm motility, whereas this relationship does not occur in the case of morphology. Finally, Table 3 shows that there is a significant and inverse relationship between percentages of aneuploidies and the percentage of typical forms, but that this significance does not occur in the case of concentration and motility.

DISCUSSION

The percentage of typical forms assessed with strict criteria was found to have no significant relationship with sperm concentration and motility. The significant and inverse relationship between morphology and sperm aneuploidy, and the lack of this significance in the case of motility and of density may be regarded as a confirmation of the absence of a significant relationship between morphology and density/motility. On the other hand, a significant positive relationship exists between the percentage of class A motile sperm and sperm concentration. Thus, sperm morphology may constitute an independent feature of the male gamete with respect to motility and concentration, and iOAT may occur via at least two independent pathways, the first mainly affecting concentration and/or motility and the second mainly affecting the morphology; that is, the postulate that iOAT patients may actually be considered a heterogeneous cohort of infertile men was hereby confirmed.

A number of tests have been carried out to study sperm DNA integrity, and some of these have not shown such an isolated relationship with sperm morphology. DNA integrity

TABLE 2. The percentage of aneuplodies of each chromosome studied in the current study (in bold) and comparison with the percentages of aneuploidies of chromosomes which are reported in literature.[a]

	Seminal characteristics of the studied population						
Chromosomes studied	NOA[b]	sOAT[c]	OAT[d]	Mt[e]	Mf[f]	Mf+Mt[g]	N[h]
1	4.8% [27]						
4			2.4% [32]				
6			0.26% [32]				
7			0.62% [32]				
8			0.46% [32]		0.98% [29]		
9			0.40% [32]				0.3% [29]
10			0.40% [32]				
11			0.26% [32]				
12		0% [29]	0.10% [32]		0% [29]		0% [29]
13	**18.6**%	**6.2**%	**2.5**%	**2.2**%	**2.0**%	**1.5**%	
		0.60% [24]	0.34% [24]			0.48% [31]	
		3.10% [14]	0.40% [32]				
		2.30% [30]					
15	**9.8**%	**3.0**%	**0.6**%	**0.7**%	**0.6**%	**0.3**%	
		2.7% [14]					
16	**10.2**%	**2.8**%	**0.7**%	**0.8**%	**0.5**%	**0.2**%	
		0.4% [14]					
17	**8.0**%	**2.7**%	**1.0**%	**0.7**%.	**0.7**%.	**0.2**%.	
	9.6% [27]	0.7% [14]	0.40% [32]				
		8.4% [31]					
18	**6.0**%	**2.4**%	**0.9**%	**0.7**%	**0.6**%	**0.2**%	0.4% [29]
	4.0% [28]	0.7% [14]	0.78% [29]	0.16% [34]	0.56% [24]	0.08% [34]	0.3% [35]
		0.92% [29]	0.22% [32]		1.28% [29]		1.0% [28]
		2.10% [30]					
21	**20.3**%	**7.1**%	**2.1**%	**1.9**%	**1.6**%	**1.4**%	1.0% [28]
	1.0% [28]	1.8% [14]	0.44% [24]	0.13% [24]		0.72% [31]	
		1.16% [24]	0.30% [33]				
		1.90% [30]	0.36% [32]				
22	**17.5**%	**6.8**%	**1.9**%	**2.2**%	**1.9**%	**1.5**%	
		2.8% [14]					
		4.12% [30]					
X	**23.9**%	**7.9**%	**3.0**%	**2.6**%	**2.3**%	**1.7**%	0.70% [11]
	2.62% [11]	1.3% [14]	0.58% [24]	0.21% [24]	1.32% [24]	0.15% [34]	1.06% [29]
	7.0% [28]	1.56% [24]	0.88% [33]	0.19% [34]	0.96% [29]	0.92% [31]	0.29% [35]
	24.2% [27]	0.90% [29]	0.30% [32]				0.80% [28]
		0.64% [30]					

[a] In each case, the percentage of aneuploid spermatozoa was computed by doubling the incidence of disomy.
[b] NOA = nonobstructive azoospermic patients.
[c] sOAT = severe oligoasthenoteratospermic patient (= sperm concentration $<5 \times 10^6$/ml).
[d] OAT = oligoasthenoteratospermic patient (= sperm concentration $>5 \times 10^6$/ml and $<20 \times 10^6$/ml).
[e] Mt = patients with isolated alterations of motility (= sperm concentration $>20 \times 10^6$/ml, WHO class A motile sperm <25%, morphology [strict criteria] >14%).
[f] Mf = patients with isolated alterations of morphology (= sperm concentration $>20 \times 10^6$/ml, WHO class A motile sperm >25%, morphology [strict criteria] <14%).
[g] Mf+Mt = patients with isolated alterations of motility and of morphology (= sperm concentration $>20 \times 10^6$/ml, WHO class A motile sperm <25%, morphology [strict criteria] <14%).
[h] N = normospermic patients (= sperm concentration $>20 \times 10^6$/ml, WHO class A motile sperm >25%, morphology [strict criteria]>14%).

provides additional information about sperm function to conventional sperm analysis [36]; however, multiple measures of genomic damage are needed to fully assess the reproductive and genetic burden in sperm [37]. DNA integrity tests do not show identical predictive values for DNA damage [24, 37–39]; they depend on the techniques and on the populations studied [24, 37–39]. Therefore, DNA integrity tests cannot be considered equivalent.

The considerable biological variability of sperm concentration, motility, and morphology, and the regression to the mean phenomenon (i.e., the spontaneous tendency to achieve normal values after multiple sperm sample examination [40]) may constitute potential biases in our paper. To overcome these problems cited in the literature, two semen samples were examined from each patient, and the mean values were used for calculations [8].

Some data from the literature may corroborate that sperm morphology seems to be relatively independent from motility and density. Morphology has been considered predictive per se of reproductive in vivo and in vitro success [18, 41–50]. Guzick sustained that when assessed with strict criteria, morphology appears to be the most informative semen measurement for discriminating between fertile and infertile men [20]. More recently, two new in vitro fertilization (IVF) methods of intracytoplasmic sperm injection (ICSI) have been introduced. The first is based on the motile sperm organellar morphology examination (MSOME) [51], and the second is based on the sperm cellular membrane birifrangence [52]; microinjection of morphologically selected sperm cells with strictly normal nuclei (defined by MSOME) or with birifrangent cellular membrane improves ICSI outcomes [51, 52]. Furthermore, testicular toxicants, such as chromium [53], nitric oxide [54], and methyl methanesulphonate [55], were found to selectively affect sperm morphology in humans and in experimental mouse models, mainly triggering PCD.

TABLE 3. Results of multivariate regression analysis which defines relationships between sperm features and the percentage of aneuploid sperm.

Relationship	Level of intersection with the Y ordinate axis	Regression coefficients	Significance of each regression coefficients of dependent variables	
			Determining factors (t)	P^a
Sperm morphology, sperm concentration, and the percentage of class A motile sperm				
Independent variable (predictor)				
Percentage of typical forms	5.2			
Dependent variables				
Percentage of class A motile sperm		0.218	1.980	NS
Sperm concentration		0.076	1.910	NS
Sperm concentration, sperm morphology, and the percentage of class A motile sperm				
Independent variable (predictor)				
Sperm concentration	5.32			
Dependent variables				
Sperm morphology		−0.009	1.119	NS
Percentage of class A motile sperm		0.126	9.112	<0.01
Sperm concentration, motility, morphology, and the percentage of aneuploid sperm				
Independent variable (predictor)				
Percentage of aneupoloid spermatozoa	3.377			
Dependent variables				
Sperm concentration		−0.004	1.001	NS
Percentage of class A motile sperm		−0.004	1.003	NS
Percentage of typical forms		−0.018	9.335	<0.01

[a] NS, not significant.

In our study, the percentage of aneuploid sperm is inversely related to morphology assessed with strict criteria. Some previous studies found a similar inverse relationship between the percentage of typical forms and the percentage of aneuploidies [24, 33, 56, 57]. Furthermore, globozoospermia [56], flagellar abnormalities [58], large-headed and multiple-tailed spermatozoa [59], and elongated-head spermatozoa [60] were linked to increased aneuploidies. The computerized cell scanning system has demonstrated the relationship between chromosomal aberrations and sperm morphology in the same spermatozoon. The incidence of chromosomal aberrations was positively linked to abnormal sperm morphology—the more severe the abnormality, the higher the incidence of aneuploidy [61]. The data found in the literature regarding relationships between sperm aneuploidies and sperm patterns are often contradictory; it has been suggested that sperm aneuploidy is linked to vitality [29], to motility [34, 62], or to concentration [30, 32, 58]. However, these studies have methodological biases; namely, few patients were tested [32, 33, 62] and/or few (one to three) autosomes + XY chromosomes were assessed [29, 34, 58] and/or the statistical analyses used were based on thresholds [17, 18, 56, 57], whereas sperm features and the percentage of aneuploid spermatozoa were continuous variables.

Normally shaped spermatozoa of OAT patients have an increased aneuploidy rate [63]. Patients with abnormal sperm parameters and a normal karyotype have an increased sex chromosome aneuploidy rate in spermatozoa and in peripheral leukocytes [64]. Chromosomes of somatic cells and of spermatozoa display archetypal modes of positioning, folding, and packaging in the decondensing nucleus [65] and in the meiotic spindle [66]. As a consequence, the analysis of aneuploidies showed different variation frequencies, the highest values of which were for sex chromosomes, followed by chromosomes 21, 13, and 22 [67]. This means that increased sperm aneuploidies should be considered to be the expression of some type of a generalized meiotic and mitotic abnormality rather than a linkage with sperm alterations [63, 64]. Consequently, the percentage of total aneuploid spermatozoa should be considered the most indicated variable to be studied for our purpose, and the more chromosomes assessed for aneuploidies, the more reliable our results should be.

Table 2 shows a great variability of the rate of aneuploid sperm according to the different authors. However, there is agreement that this variability may be due to the different populations studied and/or to the techniques that were used to assess sperm aneuplodies and/or to the number of chromosomes studied. Therefore, it has been accepted that FISH constitutes a promising test for the assessment of an infertile population [19].

More studies are needed to confirm the heterogeneity of the cohort of infertile patients identified as iOAT; however, we believe that this research may explain the contradictory efficacy of the various therapies for iOAT [1] (different studies may test different iOAT populations) and may legitimize the techniques for selecting spermatozoa for ICSI according to morphological criteria.

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